Mycoparasitism of Endophytic Fungi Isolated From Reed on Soilborne Phytopathogenic Fungi and Production of Cell Wall-Degrading Enzymes In Vitro

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Abstract Antagonism of three endophytic fungi isolated from common reed (*Phragmites australis*) against eight soilborne pathogenic fungi was investigated on potato dextrose agar by light microscopy, scanning electron microscopy, and transmission electron microscopy. Inhibitory zones were not observed. The microscopical studies suggested that the endophytes inhibit growth of soilborne pathogens by means of coiling around hyphae and, after penetration, the degradation of hyphal cytoplasm. Since penetration of hyphae seems to play a major role in parasitism, we studied the production of cell wall degrading enzymes by the three endophytes. *Choiromyces aboriginum* produced higher activities of β-1,3-glucanases compared to *Stachybotrys elegans* and *Cylindrocarpon* sp. For *C. aboriginum* and *S. elegans*, colloidal chitin was the best substrate for the induction of β-1,3-glucanases and chitinases, respectively. This result suggests that mycoparasitism by endophytes on soilborne plant pathogens can be explained by their mycoparasitic activity.

Introduction

Root rot disease, caused by soilborne pathogenic fungi including *Pythium* spp., *Rhizoctonia* spp., and *Fusarium* spp. cause widespread, serious economic loss both in greenhouse and field production systems under conditions favorable for disease development. Several studies have shown that some biological control agents (BCAs) such as *Trichoderma* spp. can reduce the incidence of root diseases caused by soilborne pathogenic fungi [33]. BCAs inhibit plant pathogens through one or more of the following mechanisms: mycoparasitism, competition for key nutrients and colonization sites, production of antibiotics, or stimulation of plant defense mechanisms [37]. Many mycoparasites such as *Chaetomium globosum* [21], *Chaetomium spirale* [16, 17], *Pythium oligandrum* [32], *Fusarium oxyporum* strain Fo47 [4], *Candida saitoana* [13], and *Verticilium lecanii* [2] have been documented since Weinding (1932) observed mycoparasitism of *Trichoderma* spp. [38].

Mycoparasites produce cell wall degrading enzymes (CWDEs) which allow them to bore holes into other fungi and extract nutrients for their own growth. Most phytopathogenic fungi have a cell wall with chitin as a structural backbone arranged in regularly ordered layers and β-1,3-glucans as a filling material arranged in an amorphous manner [10]. Chitinases and β-1,3-glucanases have been found to be involved in the parasitic interaction between *Trichoderma* species and its hosts [24]. Therefore, a study of CWDEs from mycoparasites is very essential for the evaluation of the biocontrol potential of BCAs.

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Endophytic fungi *Choiromyces aboriginum* (isolate Mü1W1C6), *Stachybotrys elegans* (isolate Mt2W1C1), and *Cylindrocarpon* sp. (isolate 5/97-12, Mt2W3C4) have been isolated from common reed (*Phragmites australis*) in the littoral of Lake Constance (Germany) [31]. Preliminary tests suggested antagonism against several soilborne fungal pathogens. Previous studies have already demonstrated that *S. elegans* isolated from soil can be a rich source of glucanolytic, chitinolytic, proteolytic, and cellulolytic enzymes [1, 35, 36]. It is capable of releasing *β*-1,3-glucanases and chitinases into a culture medium previously amended with *Rhizoctonia solani* cell wall fragments or chitin as a carbon source [35] which degrade *R. solani* cell walls [5, 6]. However, little is known about the mechanisms effective during the antagonism against soilborne pathogenic fungi and a possible production of hydrolytic enzymes by *C. aboriginum*, *Cylindrocarpon* sp., and *S. elegans* isolated from common reed.

In order to use these endophytic fungi as BCAs to control root rot diseases in the greenhouse and field, their mode of action should be studied. The main objectives of this study were to analyze the antagonisms of the three endophytic fungi *C. aboriginum*, *S. elegans*, and *Cylindrocarpon* sp. against soilborne phytopathogenic fungi and to determine which characters may contribute to their biological activities.

Materials and Methods

Fungal and Plant Material

The endophytic fungi *Choiromyces aboriginum* isolate Mü1W1C6, *Stachybotrys elegans* isolate Mt2W1C1, and *Cylindrocarpon* sp. isolate 5/97-12, Mt2W3C4 were isolated from roots of common reed (*P. australis*) in the littoral of Lake Constance (Germany). Details concerning the location and the molecular characterization have been published previously [31, 39].

The soilborne pathogenic fungi *Fusarium graminearum* isolated from wheat (*Triticum aestivum* L.), *F. oxysporum* f. sp. *perniciosum* isolated from albizzia (*Albizzia julibrissin* Durazz.), *F. oxysporum* f. sp. *vasinfectum* isolated from cotton (*Gossypium hirsutum* L.), *Gaeumanomyces graminis* var. *tritici* isolated from wheat (*Triticum aestivum* L.), *Pythium aphanidermatum* isolated from cucumber (*Cucumis sativus* L.), *Rhizoctonia cerealis* isolated from wheat (*Triticum aestivum* L.), *R. solani* isolated from seedlings of pine (*Pinus tabulaeformis* Carrb.), and *Sclerotium rolfsii* isolated from whitethorn (*Crataegus pinnatifida* Bunge) are preserved in the Lab for Resource Microorganism, Department of Plant Pathology of Shandong Agricultural University in People's Republic of China. All fungi were cultured on potato dextrose agar (PDA) plates.

Antagonism Tests In Vitro

Growth of Pathogenic Fungi in Dual Cultures

Petri dishes (60 mm) containing 6 ml of sterile PDA were inoculated with a 5 mm plug of a 1-week pure culture of three potential antagonists and eight fungal plant pathogens, respectively. The distance between the pathogen and antagonist was 30 mm. Each combination of pathogen/antagonist was repeated three times, and as negative control three petri dishes were inoculated with pathogen and a water agar plug. All petri dishes were incubated at 25°C in the dark. The experiment was replicated three times. Radial growth of each pathogenic mycelium in dual culture was recorded by measuring colony diameter at the time when they reached the margin of the dish in controls.

Light Microscope (LM)

Dual cultures of the endophytic fungi and pathogenic fungi were carried out as described above. After 2–5 days, when the two fungal colonies had grown together, the morphology of the hyphae and their behavior in the contact zone were observed with a LM at magnifications of up to 400×. Interactions between the fungi were photographed using a Zeiss Axioscop microscope and a digital camera (Canon Power Shot G7) or a laser confocal microscope (Zeiss LSM 510 META).

Scanning Electron Microscopy (SEM)

In order to further investigate the morphology of the hyphae and their behavior within the contact zone, samples (2 × 4 mm) were cut out after 2–5 days. Samples were fixed for 4–6 h in 2% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) at room temperature. Samples were rinsed thoroughly for 1–2 h with 0.2 M phosphate buffer (pH 6.8), and then dehydrated in a graded acetone series (30, 50, 70, 80, 90, and 100%), each grade for 30 min and three times for 100% acetone. Fully dehydrated samples were dried in a critical point dryer (HCP-2, Hitachi), mounted on stubs, and then coated with gold (200 nm thickness) in a sputter coater (JFC-1600, JEOL). The coated specimens were examined with a SEM (JSM-6360LV, JEOL Ltd) at 10 kV.

Transmission Electron Microscopy (TEM)

Samples were fixed in 2% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) for 3 h at room temperature.

Then samples were rinsed six times with the same buffer and post-fixed with 1% (w/v) osmium tetroxide in the same buffer for 2 h at room temperature. Samples were rinsed thoroughly with 0.2 M phosphate buffer (pH 6.8), and dehydrated in a graded acetone series, each grade for 30 min and three times for 100% acetone. Samples were infiltrated through a series of Spurr resin in acetone, and embedded in molds with fresh 100% resin and polymerized at 65°C for 36 h.

Ultrathin sections cut with a glass knife were collected on formvar coated slot grids. After drying, the grids were contrasted with uranyl acetate and lead citrate, and examined with an EM 10 CR electron microscope (Zeiss, Oberkochen, Germany) at 60 kV. Three samples per sampling time were examined with an average of 10 grid squares per sample.

Induction of Enzymes Connected With Mycoparasitism

Preparation of Fungal Cell Walls

Similar to the method of Gao et al. [16, 18], agar discs of actively growing mycelia of *R. solani* and *P. aphanidermatum* were inoculated in Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth (PDB), respectively, and incubated on a rotary shaker of 140 rev min^{-1}, at 25°C for 6 days. Fungal mycelia were collected by filtration through Xinhua no. 1 filter paper, and homogenized with pestle in a mortar and frozen in liquid nitrogen for several times. Cell wall preparation (CWP) was washed with 2% $MgCl_2$ and distilled water six times after being sonicated for 15 min. CWP was centrifuged ($10000 \times g$, 4°C, 15 min), before being lyophilized and stored at −20°C.

Culture Conditions for Enzyme Production

A synthetic medium with colloidal chitin and sucrose as carbon sources (SMCS) was used and contained (g l^{-1} of distilled water) NH_4NO_3, 1.0; K_2HPO_4, 0.87; KH_2PO_4, 0.68; $MgSO_4 \cdot 7H_2O$, 0.2; KCl, 0.2; $CaCl_2$, 0.2; colloidal chitin, 2.5; saccharose, 5.0; $FeSO_4 \cdot 7H_2O$, 0.002; $ZnSO_4 \cdot 7H_2O$, 0.002; $MgSO_4 \cdot 7H_2O$, 0.002. A basal medium (BM) was also used and contained (g l^{-1} of distilled water) peptone, 3.0; $(NH_4)_2SO_4$, 2.0; yeast extract, 0.5; KH_2PO_4, 4.0; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; Tween 20, 0.2 supplemented with 1.0 of cellulose microcrystalline (CM), or carboxymethy1 cellulose (CMC), or *P. aphanidermatum* cell walls, or *R. solani* cell walls, respectively. For the induction of chitinase, β-1,3-glucanases (endo- and exo-), β-glucosidases, filter paper activity (FPase), endo- and exo-1,4-β-D-glucanase, aliquots (50 ml) from the medium described above were put into 150 ml Erlenmeyer flask and autoclaved at 121°C, 101 kPa for 30 min. Each flask was inoculated with five agar discs (5 mm diameter) of *C. aboriginum* for 4 days or *S. elegans* and *Cylindrocarpon* sp. for 6 days old PDA and incubated with shaking at 180 rev min^{-1} at 28°C for 6 or 10 days. Culture filtrate from each flask was collected by filtration through Xinhua no. 1 filter paper and centrifuged ($15,000 \times g$) at 4°C for 15 min and the enzymes activities were determined in the supernatants.

Assays of Extracellular Enzyme Activities

Activity of chitinase was examined by the amount of the final product of *N*-acetylglucosamine (NAG) produced during the reaction. The reaction mixture consisted of 0.05 ml of the culture filtrate, 0.05 ml of 0.05 mol acetate buffer (pH 5.5), and 0.2 mg of colloidal chitin (Calculated by dry colloidal chitin). The mixture was incubated at 40°C for 4 h and the reaction was stopped by adding 0.05 ml of dinitrosalicylic (DNS) reagent (0.63% DNS, 0.50% phenol, 0.50% sodium bisulphide, and 2.14% NaOH) [29] followed by heating for 5 min. One unit (U) of chitinase activity was defined as the amount of enzyme that produced 100 μg *N*-acetyl-D-glucosamine h^{-1} under the above conditions.

The activity of β-1,3-glucanases (endo- and exo-) was measured by mixing 0.05 ml of the culture filtrate with 0.05 ml of 0.05 mol acetate buffer (pH 5.5), containing 0.1 mg of laminarin (Sigma). The mixture was incubated at 40°C for 30 min and the reducing sugar produced was determined by the method described by Miller [29]. One unit (U) of β-1,3-glucanase activity was defined as the amount of enzyme that produced 100 μg reducing sugar h^{-1} under the above conditions.

Cellulases including β-glucosidases, endo-1,4-β-D-glucanase and exo-1,4-β-D-glucanase activities as well as filter paper activity (FPase) were measured by mixing 0.05 ml of the culture filtrate with 0.05 ml of 0.05 mol acetate buffer (pH 4.8) containing 0.25 mg of salicin, CMC, 0.01 g of cotton and 50 mg of Xinhua filter paper as substrates, respectively. The mixture was incubated at 50°C for 30 min (β-glucosidases) or 60 min (endo- and exo-1,4-β-D-glucanases). The reaction was stopped by boiling, followed by adding 0.05 ml of DNS reagent and incubated 5 min in boiling bath. One unit (U) of cellulases activity was defined as the amount of enzyme that produced 100 μg reducing sugar min^{-1} under the above conditions.

Absorbance of the supernatant was measured at 540 nm by a Spectramax M2 microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Protein concentrations were measured using the method described by Lowry [26], using bovine serum albumin (Sigma) as standard. Absorbance of the supernatant was

measured at 595 nm. Specific activity was expressed in units per milligram of protein.

Results

Macroscopic Observation of Dual Cultures

Two days after inoculation, macroscopic observations revealed vigorous growth of *C. aboriginum*, *S. elegans*, and *Cylindrocarpon* sp. In the following days, mycelia of *P. aphanidermatum* and *R. solani* were overgrown by *C. aboriginum* which rapidly colonized the complete plate. Five days after inoculation the pink mycelium of *F. graminearum* had turned yellow in the contact zone. The white mycelium of *S. rolfsii* produced a yellow pigmentation after contact with *C. aboriginum*. No clear inhibition zone was observed. Similar results were obtained with *S. elegans* and *Cylindrocarpon* sp. 5/97-12.

Growth inhibition of all eight fungal pathogens was observed after the incubation with three endophytic fungi (Table 1). *Choiromyces aboriginum* had the maximum inhibitory effect on mycelial growth of *R. solani* with a reduction of 100% compared to the control, while *S. elegans* and *Cylindrocarpon* sp. were not very effective in reducing radial growth of *S. rolfsii*. Most isolates expressed mycelial growth inhibition with reductions in the range of 60–70%.

Light Microscopic Observations

Hyphae of the three endophytic fungi were easily recognized, because of their small diameter in comparison to the eight fungal pathogens. The endophytes tended to grow along the hyphae of the pathogen as soon as two colonies came into contact. We observed no differences between the endophytes with respect to the way they colonized the different pathogenic fungi. Hyphae of *S. elegans*, *C. aboriginum*, and *Cylindrocarpon* sp. coiled around hyphae of fungal pathogens and penetration was frequently observed. Here we show coils around hyphae of *R. solani* (Fig. 1a) and *P. aphanidermatum* (Fig. 1b) produced by *C. aboriginum* and hyphae of *S. elegans* (Fig. 1c) and *Cylindrocarpon* sp. (Fig. 1d) growing within hyphae of *P. aphanidermatum*.

SEM and TEM Observations

A more detailed picture of the development of coils and initiation of penetration structures was obtained by SEM and TEM experiments. Scanning electron micrographs at the contact zone revealed that hyphae of *S. elegans* growing along hyphae of *F. graminearum* branched frequently during coil formation (Fig. 2a). *Stachybotrys elegans* parasitized hyphae of *P. aphanidermatum* (Fig. 2b) and *R. solani* (Fig. 2c) in a similar way. Also *C. aboriginum* attached to *P. aphanidermatum* (Fig. 2d) and penetrated at the tip of a loop (arrowhead, Fig. 2d). Again, *C. aboriginum* parasitized hyphae of *S. rolfsii* (Fig. 2e), and hyphae of *Cylindrocarpon* sp. parasitized hyphae of *Gaeumannomyces graminis* var. *tritici* (Fig. 2f). Cell wall breakdown and hyphal disintegration were observed occasionally (data not shown). These SEM observations indicated that coiling of the antagonistic fungi around the pathogens was an early event preceding hyphal damage. A thorough investigation at the TEM level was essential to elucidate the nature of the antagonist–pathogen interaction.

Transmission electron micrographs revealed that hyphae of *S. elegans* were closely appressed to host hyphae (Fig. 3a) and started penetrating cells of *R. solani* (Fig. 3b) or *F. graminearum* (Fig. 3c) by 2–3 days after inoculation. During penetration by the antagonist (Se), the host (Fg) produced an amorphous thickening at the site of penetration which included small dark inclusions (Fig. 3c). Four days

Table 1 Inhibition of mycelial growth of phytopathogenic fungi by three endophytic fungi isolated from reed

Soilborne phytopathogenic fungi	Inhibition of mycelial growth[a] (%)		
	Choiromyces aboriginum	*Stachybotrys elegans*	*Cylindrocarpon* sp.
Fusarium graminearum	72.6 ± 1.3	83.7 ± 0.6	50.4 ± 1.3
F. oxysporum f. sp. *perniciosum*	68.2 ± 1.3	72.6 ± 5.6	61.9 ± 0.6
F. oxysporum f. sp. *vasinfectum*	68.9 ± 3.9	71.1 ± 1.9	72.6 ± 2.1
Gaeumannomyces graminis var. *tritici*	68.9 ± 2.9	77.0 ± 1.3	72.6 ± 2.6
Pythium aphanidermatum	40.7 ± 3.4	51.5 ± 0.6	78.5 ± 1.3
Rhizoctonia cerealis	55.6 ± 3.9	81.1 ± 2.9	93.3 ± 2.2
Rhizoctonia solani	100.0 ± 0	71.5 ± 1.7	93.3 ± 2.2
Sclerotium rolfsii	58.5 ± 1.3	40.1 ± 2.5	30.2 ± 0.9

Note: [a] Mean of three replicates; Count inhibition when the time reaching the margin of the dish in Control II$^+$. Inhibition (%) = $(R_0' - R_b)/R_0' \times 100$
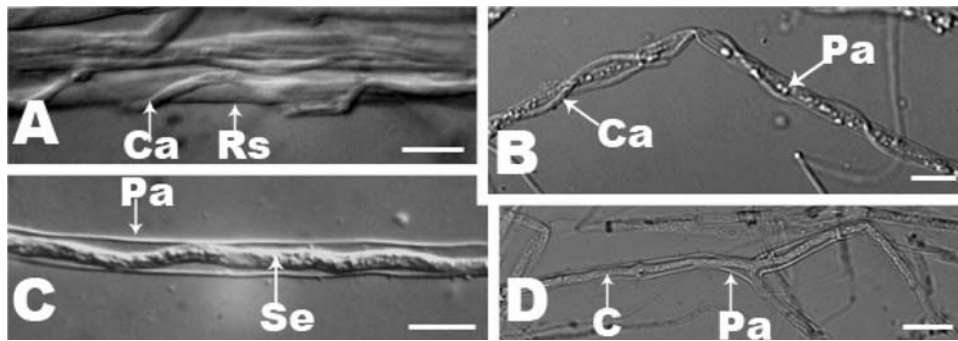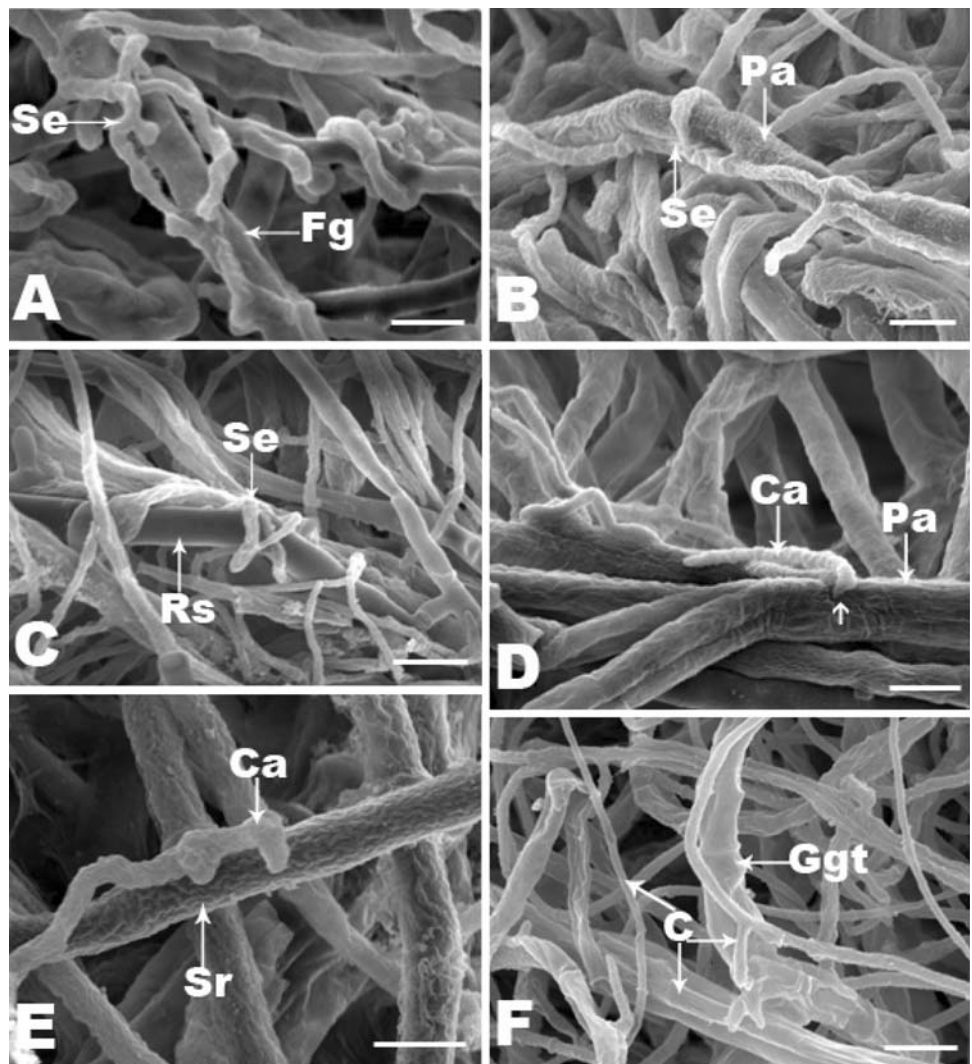
Fig. 1 Light micrographs of the hyphal interactions between three endophytic fungi and different soilborne pathogenic fungi in dual cultures. **a** Hypha of *C. aboriginum* (Ca) coiled around hyphae of *R. solani* (Rs). **b** Coiling around a hypha of *P. aphanidermatum* (Pa) by *C. aboriginum* (Ca). **c** Growth of *S. elegans* (Se) within a hypha of *P. aphanidermatum* (Pa). **d** Hyphae of *Cylindrocarpon* sp. (C) inside hyphal cells of *P. aphanidermatum* (Pa)


Fig. 2 Scanning electron micrographs of interactions between three endophytic fungi and different soilborne pathogenic fungi in dual cultures. **a** Hyphae of *S. elegans* (Se) growing along hyphae of *F. graminearum* (Fg), with frequent branches and coils. **b** Hyphae of *S. elegans* (Se) growing on hyphae of *P. aphanidermatum* (Pa). **c** Hyphae of *S. elegans* (Se) parasiting *R. solani* (Rs). **d** Penetration site of *C. aboriginum* (Ca) into a hypha (*arrowhead*) of *P. aphanidermatum* (Pa). **e** Early parasitic stages of *C. aboriginum* (Ca) in tight contact with a hypha of *S. rolfsii* (Sr). **f** Hyphae of *Cylindrocarpon* sp. (C) growing on hyphae of *G. graminis* var. *tritici* (Ggt). *Bars* = 5 μm in (**e**), 7.5 μm in (**a–d**) and (**f**)

after inoculation, hyphae of *S. elegans* had entered a hypha of *S. rolfsii* which lead to retraction of the plasma membrane and serious cytoplasmic disorganization (Fig. 3d). Five days after inoculation, hyphae of *C. aboriginum* had induced cell death of *P. aphanidermatum*, and organelles were no longer discernible (Fig. 3e). Also *C. aboriginum* attached and coiled around hyphae of *F. oxysporum* f. sp. *perniciosum*, a hemispherical wall apposition (WA) at the

Fig. 3 Transmission electron micrographs of interactions between three endophytic fungi and different soilborne pathogenic fungi in dual cultures. **a** *S. elegans* (Se) appressed to a hypha of *R. solani* (Rs) 2 days after inoculation. **b** *S. elegans* (Se) penetrated into a hypha of *R. solani* (Rs) 3 days after inoculation. **c** Prominent wall apposition (WA) in a hypha of *F. graminearum* (Fg) penetrated by *S. elegans* (Se) 3 days after inoculation. **d** *S. elegans* (Se) within a hypha of *S. rolfsii* (Sr) 4 days after inoculation. **e** *C. aboriginum* (Ca) attached to a hypha of *P. aphanidermatum* (Pa) 5 days after inoculation. **f** *C. aboriginum* (Ca) attached to *F. oxysporum* f. sp. *perniciosum* (Fo) and a wall apposition (WA) at the site of contact 3 days after inoculation. **g** Hypha of *Cylindrocarpon* sp. (C) penetrating into a hypha of *P. aphanidermatum* (Pa) 4 days after inoculation.
Bars = 1.5 μm in (**a**), (**b**) and (**d**), 1 μm in (**c**) and from (**e–g**)
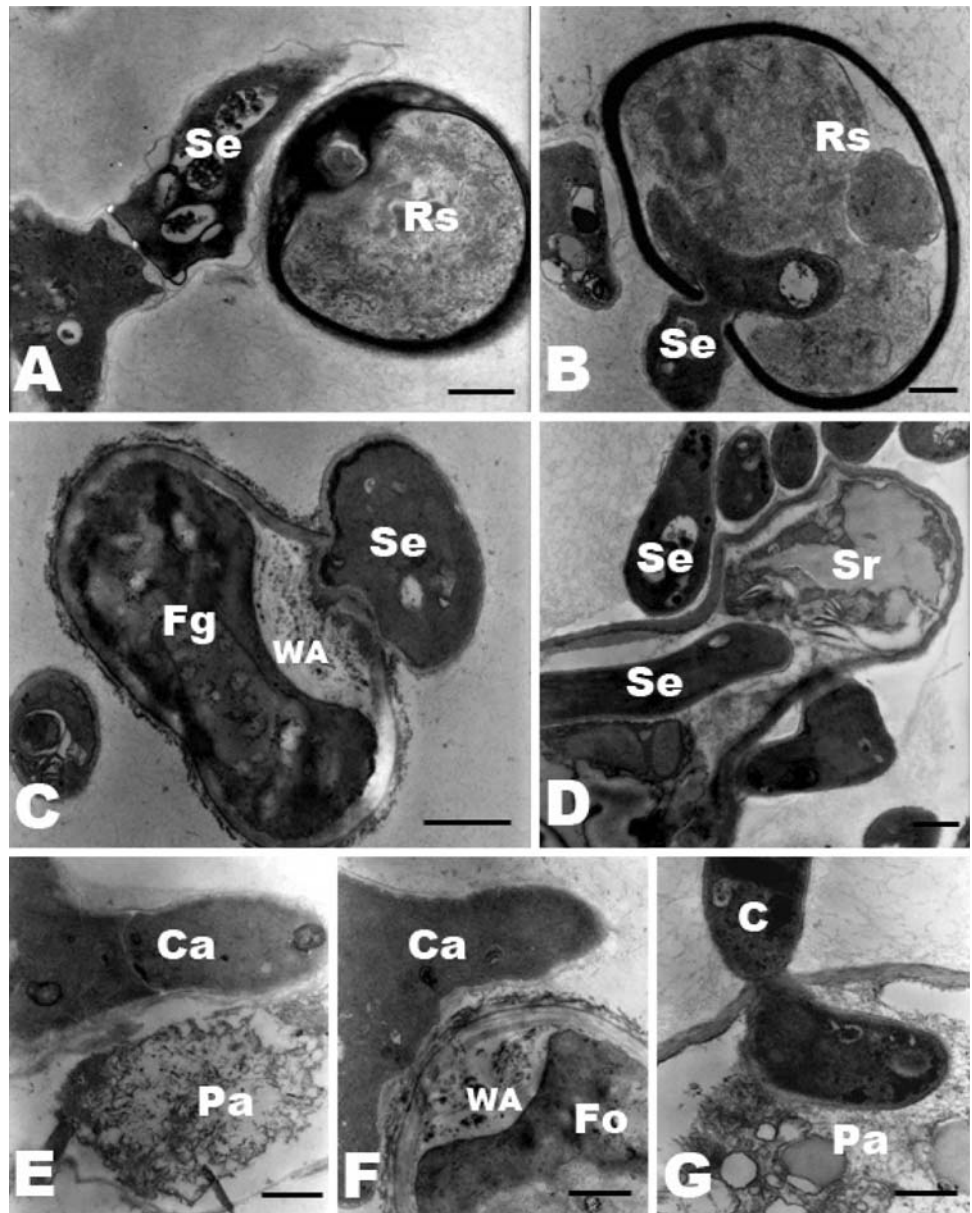
site of contact was produced (Ca) (Fig. 3f). Similarly, *Cylindrocarpon* sp. penetrated into a hypha of *P. aphanidermatum* around 4 days after inoculation (Fig. 3g). In summary, we found no differences in the penetration process and the way to degrade cytoplasmic contents of the pathogens by the three endophytes.

Enzyme Production Related to Mycoparasitism

The production of enzymes by the three antagonists in liquid media is listed in Table 2. Endo-1,4-β-D-glucanase (E.G., i.e., carboxymethyl cellulases, CMCase), exo-1,4-β-D-glucanase (cellobiohydrolase, CBH), β-glucosidases (BG) and filter paper activity (FPase), chitinases (CHI), and

β-1,3-glucanase (GLU) activities were assayed by measuring the amount of reducing sugars released from suitable substrate by means of Miller's method [29].

For all three endophytes, the activities of endo-1,4-β-D-glucanase and β-glucosidases remained at a very low level in the presence of any substrate in the medium except for the synthetic medium with colloidal chitin and sucrose and the BM with carboxymethyl cellulose as a carbon source. In contrast, the β-1,3-glucanase activities were detected at a higher level.

For *C. aboriginum* and *S. elegans*, colloidal chitin was the best substrate for the induction of β-1,3-glucanases and chitinases, respectively. Specific activities were 175.61 and 213.88 U mg^{-1} for β-1,3-glucanases, 4.21 and 25.10

Table 2 Cell wall-degrading enzymes produced by fungal endophytes isolated from reed

Isolate + medium	Cellulase activity (U mg^{-1})				CHI[e] (U mg^{-1})	GLU[f] (U mg^{-1})
	EG[a]	CBH[b]	BG[c]	FPase[d]		
C. aboriginum + Basal	0.16 ± 0.02	0.54 ± 0.03	0.09 ± 0.01	0.69 ± 0.04	1.89 ± 0.29	157.48 ± 1.68
+Basal + CMC[g]	0.15 ± 0.04	0.79 ± 0.13	0.04 ± 0.01	0.69 ± 0.04	1.57 ± 0.01	52.85 ± 0.93
+Basal + CM[h]	0.14 ± 0.03	0.43 ± 0.11	0.15 ± 0.02	0.42 ± 0.08	1.66 ± 0.25	120.37 ± 1.80
+Basal + Rs[i]	0.14 ± 0.01	0.58 ± 0.04	0.70 ± 0.01	0.54 ± 0.03	3.65 ± 0.27	133.42 ± 3.97
+Basal + Pa[j]	0.22 ± 0.01	0.90 ± 0.01	0.26 ± 0.07	0.82 ± 0.05	1.87 ± 0.02	98.25 ± 4.66
+SMCS[k]	0.13 ± 0.06	0.69 ± 0.01	0.14 ± 0.02	0.97 ± 0.06	4.21 ± 0.36	175.61 ± 5.30
S. elegans + Basal	0.15 ± 0.13	3.43 ± 0.10	0.11 ± 0.00	1.70 ± 0.09	1.85 ± 0.13	11.60 ± 1.27
+Basal + CMC[g]	0.37 ± 0.03	2.93 ± 0.03	1.32 ± 0.00	1.45 ± 0.14	1.65 ± 0.11	29.64 ± 0.81
+Basal + CM[h]	0.26 ± 0.02	1.34 ± 0.16	0.12 ± 0.02	1.23 ± 0.03	1.62 ± 0.02	12.04 ± 1.52
+Basal + Rs[i]	0.26 ± 0.02	3.19 ± 0.16	0.43 ± 0.04	1.23 ± 0.03	2.61 ± 0.22	112.85 ± 3.70
+Basal + Pa[j]	0.38 ± 0.04	1.67 ± 0.01	0.63 ± 0.11	1.30 ± 0.07	1.12 ± 0.03	33.01 ± 4.21
+SMCS[k]	4.27 ± 1.84	4.04 ± 0.76	4.86 ± 0.30	1.61 ± 0.05	25.10 ± 2.79	213.88 ± 18.79
Cylindrocarpon sp. + Basal	0.38 ± 0.00	3.77 ± 0.10	0.34 ± 0.00	1.68 ± 0.01	2.23 ± 0.03	11.41 ± 2.56
+Basal + CMC[g]	0.40 ± 0.06	3.34 ± 0.11	0.67 ± 0.05	2.38 ± 0.08	1.80 ± 0.09	26.79 ± 0.73
+Basal + CM[h]	0.19 ± 0.01	3.09 ± 0.10	0.17 ± 0.00	1.88 ± 0.10	1.85 ± 0.13	8.78 ± 2.03
+Basal + Rs[i]	0.30 ± 0.02	3.46 ± 0.15	0.26 ± 0.00	2.32 ± 0.12	1.96 ± 0.14	18.4 ± 0.85
+Basal + Pa[j]	0.42 ± 0.08	2.93 ± 0.05	0.20 ± 0.10	1.95 ± 0.10	1.68 ± 0.13	21.89 ± 2.86
+SMCS[k]	0.17 ± 0.00	3.32 ± 0.16	0.24 ± 0.01	1.46 ± 0.04	1.89 ± 0.04	8.63 ± 1.62

Notes: [a] Endo-1,4-β-D-glucanase; [b] exo-1,4-β-D-glucanase; [c] β-Glucosidases; [d] Filter paper activity; [e] Chitinase; [f] β-1,3-glucanases; [g] carboxymethyl cellulose; [h] cellulose microcrystalline; [i] Preparation of hyphal cell walls from *Rhizoctonia solani*; [j] Preparation of hyphal cell walls from *Pythium aphanidermatum*; and [k] A synthetic medium with colloidal chitin and sucrose as carbon sources

U mg^{-1} for chitinases. Fungal cell walls from *R. solani* were also superior carbon sources for the induction of β-1,3-glucanases and chitinases. Similar results by Archambault et al. [1], had shown that β-1,3-glucanases are produced during the interaction between the mycoparasite *S. elegans* and its host *R. solani*.

For *Cylindrocarpon* sp., high activities of β-1,3-glucanases were detected in the presence of carboxymethyl cellulose or fungal cell walls from *P. aphanidermatum* in the BM. Also, the activities of exo-1,4-β-D-glucanase (CBH) and filter paper activity (FPase) were somewhat higher than that of β-glucosidases (BG) and endo-1,4-β-D-glucanase (E.G.). This result suggested that *Cylindrocarpon* sp. has a strong ability to degrade the cellulose in cell wall of an oomycete such as *P. aphanidermatum*.

Discussion

The selection of effective antagonistic organisms is the first and foremost step in biological control. Most studies on antagonism have focused on a few model biocontrol strains only such as *Trichoderma* spp. [7, 8, 19], *Pythium oligandrum* [28], and *Penicillium oxalicum* [11]. Very few reports exist for *S. elegans* as biocontrol agents [1, 34]. Our investigations have provided the first evidence that mycoparasitism of endophytic *C. aboriginum*, *S. elegans*, and *Cylindrocarpon* sp. isolated from common reed contribute to antagonistic activities of endophytic fungi toward soilborne phytopathogenic fungi.

Fungal interactions in co-culture showed that *C. aboriginum*, *S. elegans*, and *Cylindrocarpon* sp inhibited mycelial growth of several soilborne phytopathogenic fungi. A similar coiling around or penetrating into the hyphae of these pathogenic fungi was confirmed through LM, SEM and TEM observations.

Our experiments on the mechanism behind this mode of parasitism show that *C. aboriginum* and *S. elegans* produce enzymes capable of degrading chitin and, β-1,3-glucan, two major cell wall compounds of many pathogenic fungi except for oomycetous fungi. In the presence of chitin or *R. solani* cell wall fragments, *C. aboriginum*, and *S. elegans* produced significant amounts of both chitinases and β-1,3-glucanases. These lytic enzymes, which are key enzymes in the lysis of cell walls of higher fungi, are produced by other organisms that are known to attack and parasitize fungi [14–16, 27, 30]. Media containing chitin or fungal cell wall fragments as carbon source supported high production of chitinases and β-1,3-glucanases, respectively. Similarly, Tweddell et al. [35] showed that the production of these enzymes in *S. elegans* is favored by chitin and fungal cell wall fragments. In addition, our study

shows that the production of β-1,3-glucanases was much higher than that of chitinases in medium containing chitin or cell wall fragments, as well as other carbon sources. These results may suggest that β-1,3-glucanases could be more important than chitinases in the degradation of *R. solani* cell walls. This is not surprising since cell walls of *R. solani* are known to contain significantly more β-1,3-glucan polymers than chitin [20]. In contrast to *C. aboriginum* and *S. elegans*, *Cylindrocarpon* sp. produced lower amounts of β-1,3-glucanases in the presence of chitin, fungal cell wall fragments.

On the other hand, *C. aboriginum* produced higher activity of β-1,3-glucanases in BM without any carbon source. This result may imply autolysis occurred under conditions of carbon energy source exhaustion. Therefore, starvation could also induce the β-1,3-glucanase. This is similar to results with the biocontrol agent *Chaetomium spirale* ND35 [16].

For cellulase including endo-1,4-β-D-glucanase (E.G., i.e., carboxymethyl cellulases, CMCase), exo-1,4-β-D-glucanase (cellobiohydrolase, CBH), β-glucosidases (BG), and filter paper activity (FPase), no correlation was found between production of enzymes from *C. aboriginum* and any substrate as a carbon source in the medium. We found that the activities of exo-1,4-β-D-glucanase and filter paper activity produced by *S. elegans* and *Cylindrocarpon* sp. were detected at a higher level in the presence of any substrates as a carbon source in the medium. This may suggest that production of exo-1,4-β-D-glucanase and filter paper activity by *Cylindrocarpon* sp. does not rely on any substrate as a carbon source in the medium. But, to some extent, production of exo-1,4-β-D-glucanase by *S. elegans* depends upon several substrates such as colloidal chitin and sucrose in the synthetic medium and *R. solani* cell wall fragments or without any carbon source in the BM.

It appears to be that no correlation was found between production of enzymes and degradability of cellulose. But, surely we observed that the endophytic fungi isolated from reed were able to penetrate and colonize the *P. aphanidermatum* hypha as seen with light and electron microscope. This may be the result of synergism of cellulases, chitinases, and β-1,3-glucanases because oomycetous fungal cell walls primarily contain cellulose, glucans (primarily β-1,4-; β-1,3-; β-1,6-; and α-1,3-glucans) and trace amounts of chitin as well.

In recent years, some genes encoding for CWDEs have been cloned and expressed in beneficial fungi to promote enzyme secretion, with the purpose to improve control. For example, transformants of *T. harzianum* that overexpressed a 33 kDa chitinase gene showed up to 200-fold greater activity and were more effective than the wild type by inhibiting the growth of the fungal pathogen *R. solani* [24]. Also transformants of *T. virens* in which β-1,3- and β-1,6-

glucanase genes were upregulated displayed much higher levels of enzyme activity than the wild-type [12]. Recent genetic evidence suggested that a 42 kDa extracellular chitinase produced by several *Trichoderma* species has a critical role in antagonism against *Botrytis cinerea* and *R. solani* [3, 22, 23, 25] and is combined with improved biocontrol efficacy [9].

SEM and TEM investigations of hyphal penetration of soilborne pathogenic fungi by three fungal antagonists suggest that extracellular metabolites such as CWDEs could be responsible for the observed penetration and degradation. The present ultrastructural investigation together with biochemical data on CWDEs activity indicate that production of these enzymes may be of great significance in the antagonistic process. Our electron micrographs support enzymatic penetration of the host hyphae and this observation supports the biochemical and molecular data on the role of enzymes in mycoparasitism.

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